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Induction of c-*jun* protooncogene expression and transcription factor AP-1 activity by the polyoma virus middle-sized tumor antigen

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ABSTRACT **Polyoma virus middle-sized tumor (PymT) antigen is required for neoplastic cell transformation by polyoma virus. We studied changes in gene expression accompanying expression of PymT in murine fibroblasts. These experiments showed that PymT differentially affects several growth-related genes. c-*jun* protooncogene expression was highly increased, whereas the expression of two growth arrest-specific genes (*gas*) was reduced, in cells transformed by PymT. Cotransfection experiments showed that the increase in c-*jun* expression resulted from elevated activity of the transcription factor AP-1 and was mediated through the phorbol 12-tetradecanoate 13-acetate response element in the c-*jun* promoter. The degree of c-Jun/AP-1 activation by different PymT mutants correlated with their transforming capability, suggesting that regulation of c-Jun/AP-1 activity may play a role in cell transformation by polyoma virus.**

Polyoma virus middle-sized tumor (PymT) antigen is required for tumorigenesis *in vivo* and for transformation of a variety of cells *in vitro* (reviewed in refs. 1 and 2). PymT is located in the plasma membrane and associates with at least three cellular proteins potentially involved in growth control: $pp60^{c\text{-}src}$ and other members of the *src* family (3–5), phosphatidylinositol 3-kinase (PI kinase) (6–8), and protein phosphatase 2A (reviewed in ref. 9). It is likely that these interactions are involved in transformation by PymT. For example, binding of PymT to $pp60^{c\text{-}src}$ increases the protein-tyrosine kinase activity of $pp60^{c\text{-}src}$, an activity that could influence growth control (10, 11).

Infection of resting cells by polyoma virus leads to expression of c-*fos*, c-*myc*, and c-*jun* protooncogenes (12, 13). These genes belong to the group of immediate early genes that are involved in regulation of cell growth (reviewed in refs. 14 and 15). Several *fos*- and *jun*-related genes have been isolated. The various Jun proteins are able to form homodimers, or heterodimers with each of the Fos proteins, constituting AP-1, a group of transcription factors that bind to the phorbol 12-tetradecanoate 13-acetate (TPA) response element (TRE), originally identified in the promoter of the human collagenase gene (refs. 16 and 17; refs. in refs. 18 and 19). TREs have been found in other TPA-stimulated genes, notably in the c-*jun* gene itself, and it has been demonstrated that c-Jun positively regulates its own expression via this TRE (20).

Another set of genes that is regulated in response to growth regulatory signals is the group of *gas* (growth arrest-specific) genes. In contrast to the immediate early genes, *gas* genes are highly expressed in growth-arrested cells, but are down-regulated in response to mitogens and serum (21).

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To study signal transduction pathways by which PymT might control cell growth, we analyzed the expression of growth-related genes in response to PymT. For this purpose, we used murine fibroblasts that were either stably transformed or transiently transfected with PymT expression vectors and measured the expression of endogenous genes or reporter constructs, respectively. We present evidence that PymT differentially regulates expression of several growth-related genes. Whereas expression of c-*jun* was highly increased in PymT-transformed cells, gas-1 mRNA, and to a lesser extent gas-2 mRNA, was decreased. The expression of other *jun* and *fos* family genes, *junB*, *junD*, *fosB*, *fra-1*, and *fra-2*, was less affected in transformed cells. Analyses of deleted promoter constructs showed that positive regulation of c-*jun* expression was mediated by transcriptional regulation through the AP-1 binding site in the c-*jun* promoter. The ability of various PymT mutants to transactivate the c-*jun* promoter correlated with their transforming potential. Since overexpression of c-*jun* is able to cause cell transformation (22, 23), increased c-Jun/AP-1 activity may play a role in transformation by polyoma virus.

MATERIALS AND METHODS

Cell Culture. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (for 3T3 cells) or fetal calf serum (for 10T½ cells). The PymT-transformed cell line and the parental 10T½ cells were generously provided by Gernot Walter (University of California, San Diego). The PymT-transformed cells were isolated by M. K. Rundell from foci produced after stable transfection of 10T½ cells with a PymT expression plasmid. The transformed cells reached saturation densities 10-fold higher than those of the untransformed cells. The transformed cells grew efficiently in soft agar (100 colonies per 500 cells seeded) and formed foci on monolayers, whereas the untransformed cells did not (no colonies or foci per 500 cells plated).

mRNA Analyses. Isolation of poly(A)$^+$ RNA, Northern blotting, and hybridization of the filter-bound RNA to radioactive probes were done as described in ref. 24. The probes used for the detection of c-jun, junB, junD, c-fos, and choA mRNA are described in ref. 25. For fosB and fra-1, we used 2.1-kilobase (kb) and 4.1-kb *Eco*RI inserts, respectively, from plasmids that were supplied by Rodrigo Bravo (Princeton University). These probes were derived from murine sequences inserted into pUC19 and Bluescript vectors, respectively. The probe for fra-2 was a 1.1-kb *Eco*RI fragment

Abbreviations: CAT, chloramphenicol acetyltransferase; PymT, polyoma virus middle-sized tumor antigen; PI kinase, phosphatidylinositol 3-kinase; TPA, phorbol 12-tetradecanoate 13-acetate; TRE, TPA response element; HSV, herpes simplex virus; tk, thymidine kinase.

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of a plasmid (26) supplied by Minami Matsui (Nippon Medical School, Kawasaki, Kanagawa, Japan). For the detection of gas mRNAs, we used 1.3-kb and 2.4-kb fragments of *gas-1* and *gas-2* plasmids (21) that were kindly provided by Lennart Philipson (European Molecular Biology Laboratory, Heidelberg).

Plasmid Constructs. The various *jun* promoter–chloramphenicol acetyltransferase (CAT) constructs have been described in refs. 20 and 27. The wild-type and mutant PymT expression vectors are described in ref. 28 and were kindly provided by Brian Druker (Dana–Farber Cancer Institute, Boston).

Transfections and CAT Assays. NIH 3T3 cells were seeded at a density of 500,000 cells per 100-mm Petri dish 24 hr prior to transfection. Transfections were carried out using the 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid/calcium phosphate precipitation method (29). After incubation for 16–20 hr at 37°C in 3% CO_2, the cells were rinsed with Tris-buffered saline and refed with DMEM supplemented with 0.5% calf serum. After an additional 24 hr of incubation, the cells were harvested, and the CAT activity was determined (30). Differences in transfection efficiency were corrected by using β-galactosidase activity: 2 μg of a Rous sarcoma virus–β-galactosidase expression plasmid was included with the reporter plasmid, and the total amount of DNA in the transfection mixtures was adjusted to 20 μg with pGEM-4.

RESULTS

Differential Expression of Growth-Related Genes in Normal and PymT-Transformed Fibroblasts. To analyze changes in gene expression accompanying PymT expression, we compared mRNA levels of several growth-related genes in PymT-transformed cells and the nontransformed parental cell line 10T½. Fig. 1 shows that the levels of gene expression were affected differentially by transformation. Whereas the level of c-jun mRNA was highly elevated in PymT-transformed cells, junD mRNA was only moderately increased, and junB mRNA was decreased (Fig. 1*A*). Analyses of *fos* family gene expression also revealed differential regulation. Whereas c-fos mRNA was slightly elevated, fosB mRNA was not changed, and fra-1 and fra-2 mRNAs were significantly lower in the transformed cells (Fig. 1*B*). We also measured the expression of two *gas* genes. The mRNA levels of both genes, *gas-1* and *gas-2*, were lower in the transformed cells (Fig. 1*C*). The mRNA levels of *choA*, a gene with unknown function isolated from Chinese hamster ovary cells, were slightly lower in the transformed cells. (This gene is generally used as a control for the amounts of RNA analyzed and was used as such in these experiments.) Taken together, these results indicate that expression of several growth-related genes is differentially regulated in PymT-transformed cells. Since we found a strong increase in c-jun mRNA levels in transformed cells and since c-*jun* has been shown to transform cells, we decided to analyze expression of this gene in more detail.

To confirm that the elevated levels of c-jun mRNA were reflected by an increased amount of c-Jun protein in PymT-transformed cells, we compared c-Jun protein levels in the normal and transformed cell lines. Immunostaining with anti-c-Jun antibodies showed a high level of c-Jun protein in the transformed cells compared to the parental cells (Fig. 2).

Increased Transcription Factor AP-1 Activity in PymT-Transformed Cells. To study the mechanism of regulation of gene expression by PymT in more detail, we performed transient transfection analyses with PymT in normal 10T½ cells and measured the transcriptional activities of growth-related gene promoters. The c-*jun* and c-*fos* promoters fused to the bacterial CAT gene were used as reporter constructs

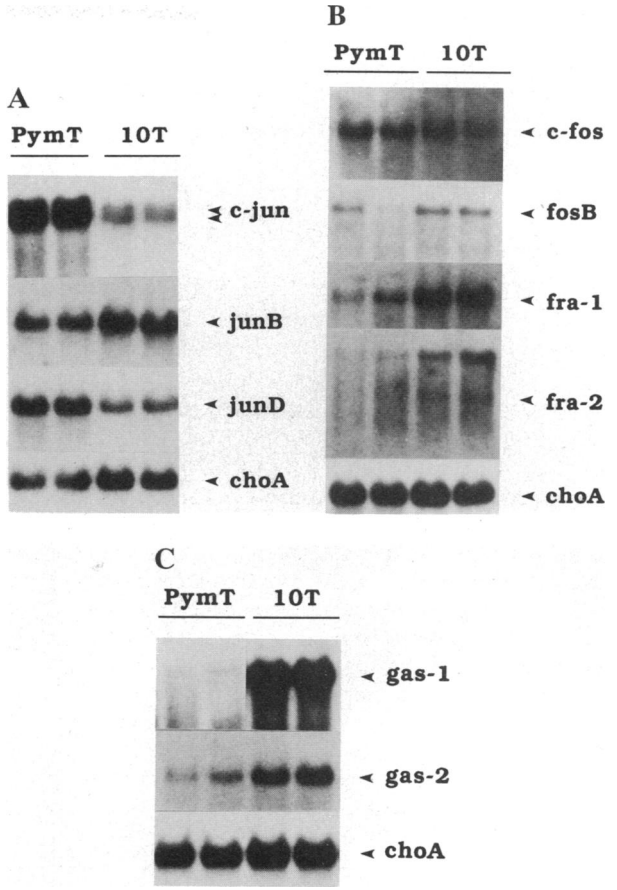

FIG. 1. mRNA levels in normal and PymT-transformed fibroblasts. Nontransformed (10T) and PymT-transformed (PymT) 10T½ fibroblasts were grown in 10% fetal calf serum/DMEM, and poly(A)$^+$ RNA was isolated essentially as described (24). Five micrograms of poly(A)$^+$ RNA from two independent experiments was separated on a formaldehyde/agarose gel and transferred to a nylon membrane by Northern blotting. Filters were hybridized to probes from *jun* family genes (*A*), *fos* family genes (*B*), and *gas* family genes (*C*) as described (24). Several filters were processed in parallel, and after the first hybridization they were stripped and rehybridized to a second probe. As a control, all filters were hybridized with a probe for choA in a third round of hybridization (not shown for all filters). To further control for the same amounts of RNA loaded in each lane, gels were stained with acridine orange before transfer (not shown). The arrowheads indicate the correct sizes of the respective transcripts as described in the relevant literature (14, 15). The significance of the weaker signal in the second lane of fosB mRNA is unclear, since in repetitions we do not see a difference in fosB mRNA between the two cell types.

and were transfected into 10T½ cells with and without a PymT expression vector. Fig. 3 shows that the CAT activities in c-*jun*–CAT- and c-*fos*–CAT-transfected cells were 3.9-fold and 8.2-fold higher when the cells were cotransfected with the PymT vector (as noted in the *Discussion*, negative autoregulation by c-*fos* could account for the smaller effect observed in PymT-transformed cells described above). This result suggests that PymT affects signal transduction pathways that regulate the activity of the c-*jun* and c-*fos* promoters. The herpes simplex virus (HSV) thymidine kinase (tk) promoter fused to the CAT gene (tk–CAT) was only slightly affected by PymT (Fig. 3), confirming that PymT does not activate all promoters.

The levels of c-jun mRNA were also elevated in PymT-transformed NIH 3T3 cells (data not shown). To assess the role of various sequence elements of the c-*jun* promoter in conferring the response to PymT, we used a series of 5′ deletion constructs of the c-*jun* promoter fused to the CAT

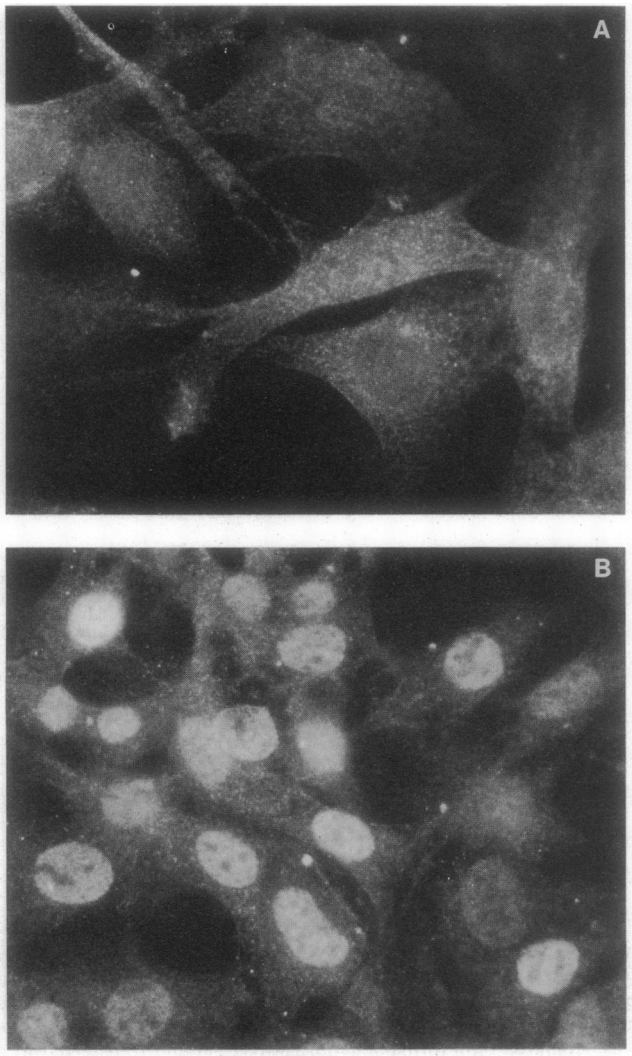

FIG. 2. c-Jun protein levels in normal and PymT-transformed fibroblasts. Untransformed (*A*) or PymT-transformed (*B*) 10T½ fibroblasts were grown on glass coverslips in 10% fetal calf serum/DMEM. Cells were fixed and then stained with anti-c-Jun antibody (Ab-2; Oncogene Sciences, Mineola, NY) as described (24). The nuclear staining seen in the PymT-transformed cells is indicative of the presence of c-Jun protein.

gene. We performed transient transfections in NIH 3T3 cells and measured the effects of PymT on the various promoter constructs. The results are summarized in Table 1. A construct comprising 1600 base pairs of 5′ upstream sequences plus 740 bp of 5′ untranslated sequences of c-*jun* (−1600/+740) was induced 4.9-fold by PymT (conversion of substrate: 15.8% in the absence of PymT, 78% in the presence of PymT). Deletion of the AP-2 sites and a low-affinity AP-1 site (construct −1600/+170) had no significant effect on the enhancement of expression by PymT. In contrast, mutation of a high-affinity AP-1 binding site, which is also the element necessary for positive autostimulation (construct −1600/+170ΔAP-1), abolished the induction by PymT. Construct −1600/+170Δ−132/−68, which contained deletions of an SP-1 site and a CAAT box transcription factor site in addition to the mutated AP-1 site, was also nonresponsive to PymT.

Other constructs, containing essentially or only the high-affinity AP-1 site (−132/+170 and −79/+170, respectively), were induced by PymT. Corresponding constructs of these minimal promoters containing a mutation in the AP-1 site that abolishes AP-1 binding (−132/+170ΔAP-1 and −79/+170ΔAP-1) were not induced by PymT. Furthermore, a

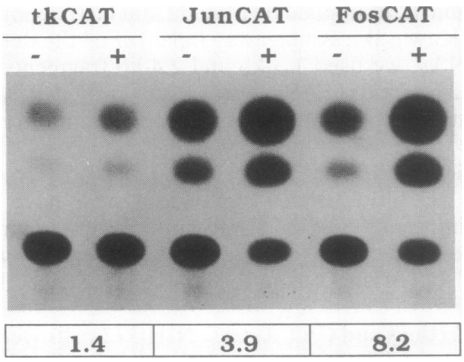

FIG. 3. CAT activity of different reporter constructs in the presence or absence of PymT. CAT reporter plasmids under the control of either the HSV tk (tkCAT), the c-*jun* (JunCAT), or the c-*fos* (FosCAT) promoter were transiently cotransfected with (+) or without (−) a PymT expression vector. Then CAT assays of cell lysates were performed as described in *Materials and Methods*. Numbers at the bottom show the fold induction of the respective CAT constructs in the presence of PymT. Repetitions of this experiment yielded similar results.

construct containing only the high-affinity AP-1 binding sequence of the c-*jun* promoter inserted upstream of the HSV tk promoter–CAT construct (1×TRE^{Jun}) was induced by PymT, whereas an equivalent construct containing a mutated TRE sequence (1×TRE^{Mut}) was not induced.

Since the sequence of TREs differs in certain promoters, such as in the collagenase gene (the c-*jun* sequence is 5′-GTGACATCAT-3′; the collagenase sequence is 5′-ATGAGTCAG-3′), a collagenase TRE was analyzed to determine whether this element was stimulated by PymT. A construct with a pentameric collagenase TRE upstream of the HSV tk promoter (5×TRE^{coll}) was induced by PymT.

These results demonstrate that the AP-1 binding site at −70 is both necessary and sufficient for transcriptional stimulation of the c-*jun* gene by PymT. We conclude that PymT is able to stimulate expression of target genes via elevation of c-Jun/AP-1 activity.

Induction of c-*jun* by PymT Mutants Correlates with Their Transforming Ability. To study the potential contribution of increased c-Jun/AP-1 activity to PymT-induced cell transformation, we compared the ability of various PymT mutants to stimulate c-*jun* promoter activity and compared this to

Table 1. The c-*jun* TRE is necessary and sufficient for PymT transactivation

Construct	Fold induction by PymT
−1600/+740	4.9
−1600/+170	4.8
−1600/+170ΔAP1	1.3
−1600/+170Δ−132/−68	0.9
−132/+170	5.0
−132/+170ΔAP1	1.3
−79/+170	5.2
−79/+170ΔAP1	1.1
1×TRE^{Jun}	3.7
1×TRE^{Mut}	1.0
5×TRE^{coll}	12.5
PBL CAT-4	1.2

NIH 3T3 cells were transiently transfected with 3 μg of c-*jun*–CAT reporter construct, with or without 2 μg of the PmyT expression vector. Cells were harvested, and CAT assays were performed as described in *Materials and Methods*. The fold induction is the amount of CAT activity in PymT-cotransfected cells divided by the CAT activity in cells transfected with the reporter construct alone. Repetitions of this experiment yielded similar results.

Table 2. Transactivation of the c-*jun* promoter by PymT mutants

Vector	Transformation: Agar growth	Transformation: Foci	Kinase activity: PI	Kinase activity: pp60$^{c\text{-}src}$	Fold activation of c-*jun*–CAT
wt	+	+	+	+	8.3
MTdl-1015	−	+/−	+	+	4.0
MTdl-23	−	(−)	(−)	+	1.5
MTNG-59	−	−	−	−	1.5

NIH 3T3 cells were transfected with 3 μg of −132/+170 c-*jun*–CAT, together with 4 μg of wild-type (wt) or mutant PymT expression vectors. CAT assays were performed as described in *Materials and Methods*. The fold activation is the amount of CAT activity in PymT-cotransfected cells divided by the CAT activity in cells transfected with the reporter construct alone. Repetitions of this experiment yielded similar results. The properties of the mutants are described in ref. 31. +/−, slow growing foci on BALB/3T3 and NIH 3T3 cells; (−), slight or no activity.

their transforming ability, as reported (31). We used the following PymT mutants, which differ in transforming ability, association with pp60$^{c\text{-}src}$, and association with PI kinase: MTdl-1015, MTdl-23, and MTNG-59. The wild-type and mutant PymT expression vectors were used to transiently cotransfect NIH 3T3 cells with the −132/+170 c-*jun*–CAT reporter plasmid, and CAT enzyme activity was determined. The properties of the mutants (adapted from ref. 31), and the results of the transfection experiments, are summarized in Table 2. The ability of the PymT mutants to induce the c-*jun* reporter plasmid correlated with their transforming ability: wild-type PymT showed the strongest induction, MTdl-1015 induced less well, and MTdl-23 and MTNG-59 showed little induction.

DISCUSSION

The PymT antigen plays a key role in cell transformation and tumorigenesis by polyoma virus. To examine the possibility that PymT affects growth control by manipulating host cell gene expression, we studied the response of several growth-related genes to the expression of PymT. Our results show that the expression of the c-*jun* protooncogene is strongly increased in PymT-transformed cells and also in cells co-transfected with PymT expression vectors and c-*jun* reporter plasmids. One component of this effect is enhanced transcription of the c-*jun* gene mediated via the AP-1 binding site (TRE) in the c-*jun* promoter. Since the c-Jun protein itself participates in AP-1 formation, it is likely that PymT increases c-*jun* expression by stimulation of this autoregulatory loop. Although *junB* and *fra-2* expression are only slightly decreased in PymT-transformed cells, this down-regulation could further contribute to c-*jun* autostimulation, since it has been demonstrated that JunB and Fra-2 are negative regulators of c-Jun activity (19, 32).

Expression of other genes whose gene products participate in AP-1 formation is affected less strongly in PymT-transformed cells. Although we did not evaluate the contribution of each of these proteins to AP-1 activity, it seems clear from our results that the net effect is an increase in AP-1 activity. Activation of c-*fos* transcription by PymT could also contribute to this increase. PymT activates multiple response elements in the c-*fos* promoter (G. M. Glenn and W.E., unpublished data). However, the strong induction of c-*fos* promoter activity that we found by transient cotransfections (Fig. 3) is not completely reflected in increased c-fos mRNA levels in stably transformed cells (Fig. 1*A*). This could be explained by an initial induction of c-*fos* (that is detectable in short term assays) that later is reduced by the effects of negative autoregulation of c-*fos* (33, 34).

How might PymT influence transcription of growth-related genes? Elevated activity of PEA 1 (AP-1) has been observed previously in cells transformed by several oncogenes, including PymT (35). In that study it was observed that PEA 1 activity was independent of serum concentration in cells transformed by PymT but was dependent on serum concentration in untransformed cells. In our experiments the increase in c-Jun and the decrease in *gas* gene transcripts, in cells transformed by PymT, are unlikely to be caused by differences in the growth state of the normal and transformed cells, because both cell types were actively growing in medium containing high levels of serum when the assays were performed. However, PymT might confer an increased sensitivity to serum factors that influence transcription via growth factor receptors, thereby affecting the levels of c-*jun* and *gas* gene expression.

Alternatively PymT might affect intracellular mediators of signal transduction by affecting PI kinase activity. This explanation would be consistent with the observation that the PymT mutants that fail to bind PI kinase show decreased levels of c-*jun* activation. PymT might also exert effects on transcription by altering the phosphorylation of cellular components. Members of the *src* family of protooncogenes are obvious candidates for mediating effects on phosphorylation, but the decreased activation of c-*jun* by the PymT mutants dl-23 (which activates pp60$^{c\text{-}src}$) and NG-59 (which does not) is not consistent with this suggestion. Effects on phosphorylation might also be brought about by protein phosphatase 2A, which binds PymT (reviewed in ref. 9). Various mechanisms by which PymT might influence transcription are not mutually exclusive, and more than one may play a role.

Our results differ somewhat from findings reported by Rameh and Armelin (36). They observed that BALB/3T3 cells transformed by PymT become independent of platelet-derived growth factor for growth and that the immediate early genes c-*myc* and *JE*, but not c-*fos* or c-*jun*, are constitutively expressed in PymT transformants. This discrepancy could be due to the use of different cell lines or to differences in experimental design. For example, they analyzed mRNA levels in transformants after serum starvation, whereas we used transformed cells growing in high serum concentrations.

The increased expression of c-*jun* and the decreased expression of *gas* genes, in response to PymT, together with the correlation between activation of c-*jun* and the transforming potential of PymT mutants, suggest that regulation of growth-related genes may play an important role in neoplastic cell transformation by polyoma.

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